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A protein methylesterase involved in bacterial sensing

(chemotaxis/*S*-adenosylmethionine/*Salmonella typhimurium*/*Escherichia coli*)

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ABSTRACT **A protein methylesterase has been identified in soluble extracts of *Salmonella typhimurium* and *Escherichia coli*. This enzyme catalyzes the hydrolysis of γ-glutamyl methyl ester residues from membrane-bound 60,000-molecular weight proteins that are essential for chemotaxis. Analyses of methylesterase activity in a variety of chemotactically defective strains suggest that the methylesterase is a product of the *cheX* gene in *Salmonella* and the *cheB* gene in *E. coli*. In addition, the *cheT* gene product in *S. typhimurium* seems to play a role in expression of methylesterase activity. Mutant strains lacking the protein methylesterase tumble incessantly in the absence of attractant gradients. This behavior is the converse of that shown by mutant strains defective in methyltransferase activity, which swim smoothly in the absence of repellent gradients. This finding indicates that reversible methylation acts as a control mechanism and that both a methyltransferase and a protein methylesterase are instrumental in bacterial sensing.**

Methylation reactions are extensive in biological systems. They are known to be important in the modification of nucleic acids, proteins, carbohydrates, and lipids (1). In essentially all of these reactions the modification remains for the lifetime of the modified molecule. Irreversible modification is of course known in peptide activation, as in processing to convert a zymogen to a zymase or a prohormone to a hormone (2). On the other hand, covalent modification is usually reversible in reactions such as phosphorylation, adenylylation, and ADP-ribosylation. Until recently, no catalyzed reversible attachment and removal of methyl groups has been observed, but from recent studies on bacterial chemotaxis, such a reversible protein methylation appears likely.

Tactic responses of the bacteria *Escherichia coli* and *Salmonella typhimurium* to chemicals in their environment are mediated by a relatively simple sensory response system. Receptor proteins at the cell surface detect trace levels of extracellular chemicals (3). Migration of bacteria towards favorable environmental conditions is effected by control of tumbling frequency (4, 5), which is in turn generated by reversal of flagellar rotation (6, 7). From genetic studies it has been shown that at least nine polypeptides are involved in the signal transduction process (8), and, in addition, there are at least two intrinsic membrane proteins of 60,000 molecular weight that function in conjunction with the receptor proteins (9–11).

So far the function of only one of the nine polypeptides has been identified: the *cheR* gene product is involved in the expression of a methyltransferase that catalyzes the transfer of methyl groups from *S*-adenosylmethionine to the 60,000-molecular weight membrane proteins (12). The products of the reaction are γ-glutamyl methyl ester residues in the membrane proteins, and *S*-adenosylhomocysteine (13, 14). Mutant strains lacking either the methyltransferase or the 60,000-molecular weight membrane proteins (12, 15), and wild-type cells deficient in methionine (16, 17) or *S*-adenosylmethionine (18, 19) are defective in chemotaxis. Furthermore, levels of methylation of the membrane proteins change rapidly when cells are exposed to chemical stimuli: attractants cause increases in methylation and repellents cause decreases (20).

Protein carboxyl methylation has also recently been found in mammalian systems (21) and has been associated with leukocyte chemotaxis (22).

In this paper, we describe the discovery of a protein methylesterase that functions in chemotaxis and the relationship of this enzyme to chemotactically defective strains of *E. coli* and *Salmonella*.

MATERIALS AND METHODS

S-Adenosyl-L-[*methyl*-^{3}H]methionine (12.6 Ci/mmol) was obtained from Amersham. *S*-Adenosyl-L-methionine was obtained from Sigma.

Except for ST324 and ST325, all the *S. typhimurium* strains used in this study have been described previously (23) or were obtained from B. A. D. Stocker (SL1509, SL1507, SL2516, SL4041). ST324 and ST325 were derived from *S. typhimurium* ST314 by A. DeFranco. *E. coli* strains were obtained from J. S. Parkinson (University of Utah).

Cells were grown in nutrient broth (Difco) containing 5 g of NaCl per liter. They were harvested during exponential growth at a cell density of approximately 1 g wet weight of cells per liter of culture and collected by centrifugation for 20 min at 8000 × *g*. Cells were resuspended in 1–3 vol of 0.1 M sodium phosphate/1.0 mM EDTA, pH 7.1, and disrupted by sonication with a model W220F sonicator (Heat Systems–Ultrasonics, Inc.). Intact cells and large cell fragments were then removed by centrifugation at 12,000 × *g* for 20 min. Extracts were either assayed directly for methylesterase activity or separated into soluble and membrane fractions by centrifugation at 100,000 × *g* for 2 hr in a Beckman model L ultracentrifuge. The resulting membrane pellets were resuspended in sodium phosphate/EDTA buffer to a final concentration of approximately 25 mg of membrane protein per ml of suspension. The supernatant material contained from 15 to 50 mg of protein per ml.

Radioactive-methylated membranes were prepared to be used as substrates in the protein methylesterase assay. A strain deficient in protein methyltransferase activity (ST1038) (12) was used to ensure the presence of unmodified carboxyl groups in the initial membrane preparation. These preparations were then treated with tritium-labeled *S*-adenosylmethionine and a soluble cytoplasmic extract of wild-type strains that contained methyltransferase activity (12). The final concentrations of membrane and soluble proteins were 2 and 5 mg/ml, respectively. After a 60-min incubation at 30° the methylated membranes were removed by centrifugation at 100,000 × *g*, washed three times by repeated centrifugation and resuspension in sodium phosphate-EDTA buffer, and finally resuspended

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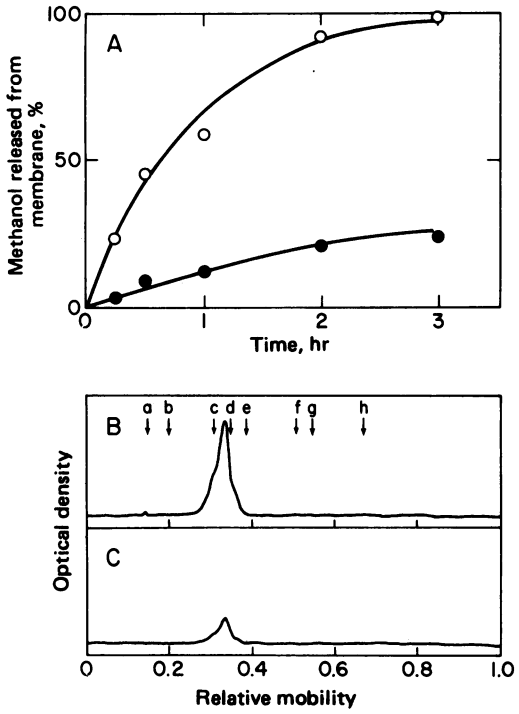

FIG. 1. Hydrolysis of methylated 60,000-dalton proteins catalyzed by soluble extract of *S. typhimurium* ST1. Methylated ST1038 membranes containing 135 pmol (247,000 cpm) of carboxyl [^{3}H]-methyl groups were incubated at 30° in the presence (○) or absence (●) of 31 mg of *S. typhimurium* ST1 soluble extract protein in a final volume of 1.0 ml of 0.1 M sodium phosphate/1.0 mM EDTA, pH 7.1. Aliquots were removed from each suspension at the indicated times and analyzed for methanol released by the ethanol precipitation assay as described in *Materials and Methods* (*A*) or boiled in 2% sodium dodecyl sulfate for 5 min and analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis as described previously (12). The stained gels were treated with 2,5-diphenyloxazole (PPO) and autoradiographed according to the method of Bonner and Laskey (25). The autoradiogram was scanned using white light in a Transidyne RFT scanning densitometer set in the linear mode. The arrows in *B* indicate the positions of the following molecular weight marker proteins: a, β-galactosidase (116,000); b, phosphorylase (94,000); c, bovine serum albumin (68,000); d, catalase (60,000); e, glutamate dehydrogenase (56,000); f, alcohol dehydrogenase (41,000); g, glyceraldehyde-3-phosphate dehydrogenase (36,000); and h, *S. typhimurium* histidine-binding protein (25,000). In *B* are shown results obtained with membranes incubated in buffer for 0–3 hr or in soluble extract at time zero. In *C* are results with membranes incubated in soluble extract for 3 hr. Membranes incubated in soluble extract for shorter times gave intermediate results (data not shown).

in the same buffer to a protein concentration of approximately 2 mg/ml.

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of the methylated membranes showed that the only proteins labeled comigrated with the 60,000-molecular weight proteins previously identified with chemotaxis. When the same incubation was carried out including tritiated S-adenosylmethionine but utilizing soluble extract from ST1038 (the methyltransferase-deficient mutant), no labeling of this protein fraction was observed.

Methylesterase activity was measured by using methylated membranes as substrate. The membranes were incubated with cell extracts at 30°, and at various intervals 0.1-ml aliquots were added to 0.9 ml of cold ethanol. The resulting precipitate was removed by centrifugation for 30 sec in a Beckman Microfuge and the supernatant was assayed for radioactivity. It was shown

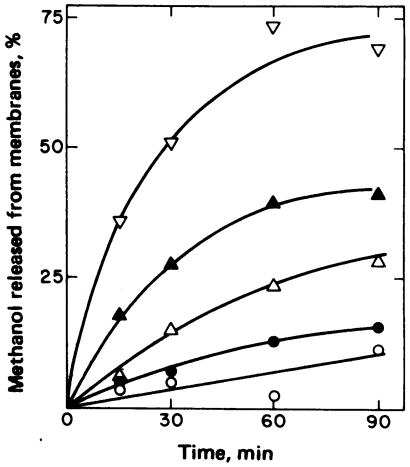

FIG. 2. Methylesterase activity as a function of concentration of cell extract protein. Methylesterase was assayed with various amounts of *S. typhimurium* ST1 extract. The final levels of soluble protein in mg per ml of reaction mixture were: ▽, 40.7; ▲, 16.3; △, 8.1; ●, 4.1; and ○, 0.0. Each reaction mixture initially contained 18.2 pmol of carboxyl [^{3}H]methyl groups in 0.22 mg of membrane protein in a volume of 0.6 ml.

that the radiolabel introduced into the protein fraction of the membranes could be entirely released by incubation for one hour at 30° in 0.1 M NaOH and that it appeared entirely as methanol.

Radioactivity was determined by liquid scintillation spectrometry in a Packard Tri-Carb spectrometer with Handifluor scintillation fluid. Protein was determined by the method of Lowry *et al.* (24), modified by first precipitating the protein in 7.5% trichloroacetic acid.

RESULTS

Demonstration of Demethylation of Membrane Protein. Protein methylesterase activity was determined by incubating extract from wild-type cells with specifically labeled membranes. As described above, the membranes were methylated in the γ-glutamyl residue of 60,000-molecular weight chemotaxis proteins by the methyltransferase enzyme. When these labeled membranes are incubated with extracts of wild-type *S. typhimurium* cells, the label was released as tritiated methanol within a few hours (Fig. 1). The appearance of radioactive methanol was shown to be concomitant with the decrease of tritium label in the 60,000-molecular weight membrane proteins. Little hydrolysis occurred in controls in which the methylated membranes were incubated in buffer or in the presence of boiled extract.

The rate of methyl ester hydrolysis was found to be first order in both the concentration of methylated membranes (in the range from 0.04 to 2 mg of protein per ml) and in the concentration of the cell-free extract (Fig. 2.)

The methylesterase activity was due to a soluble component of the extract, as shown by the fact that there was no significant loss of activity when membranes were removed from extracts by centrifugation at 100,000 × *g* for two hours. Moreover, no low molecular weight factor appeared to be required because extensive dialysis did not affect the ability of extracts to catalyze the methylesterase reaction. These results showed that the methylesterase activity was caused by a cytoplasmic component of wild-type *S. typhimurium* cells. The activity was found to be heat labile and unaffected by dialysis, properties of a protein molecule.

Table 1. Methylesterase activities in nonchemotactic (*che*$^-$) strains of *Salmonella typhimurium*

Strain	Relevant genotype	Methylesterase activity,* %	Nongradient swimming behavior†
ST1	Wild type	100	Random
ST1002	*cheP*$^-$	60	Smooth
ST1001	*cheQ*$^-$	57	Smooth
ST1038	*cheR*$^-$	45	Smooth
ST108	*cheS*$^-$	125	Smooth
ST171	*cheT*$^-$	1	Tumbly
ST155	*cheU*$^-$	66	Random
SL2516	*cheV*$^-$	74	Smooth
ST1024	*cheW*$^-$	87	Smooth
SL4041	*cheX*$^-$	1	Tumbly

* Methylesterase activity was measured as described in *Materials and Methods*. The soluble extract of *S. typhimurium* ST1 catalyzed hydrolysis of 3.6% of the carboxyl methyl groups in specifically methylated ST1038 membranes per mg of soluble extract protein per ml per hr.

† Swimming behavior in the absence of attractant or repellent gradients. "Random" corresponds to alternating periods of smooth swimming and tumbling.

Identification of the Protein Methylesterase with a Gene. The nine classes of motile but generally nonchemotactic mutant strains previously associated with chemotaxis in *Salmonella* (8) were each tested in the methylesterase assay. Soluble extracts of each of the strains were incubated with tritium-labeled membranes, and the radioactivity released was evaluated as shown in Table 1. Of the strains with mutations in the nine chemotaxis genes, only the *cheT*$^-$ and *cheX*$^-$ strains lacked methylesterase activity.

Double and triple mutants were also examined for methylesterase activity. The extract of a mutant defective in *cheX*, *cheQ*, and *cheT* did not catalyze demethylation of the 60,000-dalton methylated membrane proteins, but, on the other hand, a mutant strain defective in both *cheQ* and *cheT* had significant activity (Table 2). Thus, in *Salmonella* all the *cheX*$^-$ strains were methylesterase deficient, whereas *cheT*$^-$ strains gave mixed results. All other strains had normal or essentially normal amounts of the methylesterase activity.

In *E. coli*, eight genes for general nonchemotaxis have been identified (11, 26, 27); strains defective in each of these genes were therefore assayed for methylesterase activity. Only the *cheB*$^-$ mutant lacked activity (Table 3). This is a particularly interesting result because genetic studies of DeFranco *et al.** have indicated that the *cheX* gene of *Salmonella* corresponds to the *cheB* gene of *E. coli*. Taken together, these results strongly indicate that the gene product of the *cheX* gene in *Salmonella* and the product of the corresponding *cheB* gene in *E. coli* are needed for expression of methylesterase activity, and the genes each probably code for a protein involved in this process.

The results concerning the *cheT*$^-$ gene product are not as definitive. The corresponding gene in *E. coli* is *cheZ*, and *cheZ*$^-$ strains have methylesterase activity, while some *cheT*$^-$ strains of *Salmonella* have no activity. In studies of genetic complementation, Parkinson (27) has observed poor complementation between certain *cheB*$^-$ and *cheZ*$^-$ *E. coli* strains. This led him to hypothesize (11) that these gene products interact in some way. The mixed results reported here with the *cheT* gene product in *Salmonella* and the *cheZ* gene product in *E. coli* would be consistent with such a hypothesis.

* A. L. DeFranco, J. S. Parkinson, and D. E. Koshland, Jr., unpublished results.

Table 2. Methylesterase activities in various *Escherichia coli* and *Salmonella typhimurium* strains

Strain	Relevant genotype	Methylesterase activity,* %
S. typhimurium		
ST1	Wild type	100
SL1509	*flaC*$^-$	8
SL1507	Δ*flaC–motC*	1
ST324	*cheQ*$^-$, *cheT*$^-$	47
ST325	*cheX*$^-$, *cheQ*$^-$, *cheT*$^-$	3
E. coli		
RP437	Wild type	100
RP4368	*tsr*$^-$	317
RP4324	*tar*$^-$	277
RP4372	*tar*$^-$, *tsr*$^-$	187

* Wild-type activities are those shown in Tables 1 and 3.

Tests for Methylesterase Activity in Other Mutant Strains. Extracts derived from *fla*$^-$ *Salmonella* strains did not catalyze demethylation of the methylated 60,000-dalton membrane proteins (cf. Table 2). *S. typhimurium* mutant strains unable to synthesize complete flagellar basal structures (*fla*$^-$ mutants) are deficient in methyltransferase activity and also lack the 60,000-dalton membrane proteins (S. Clarke, K. T. Sparrow, and D. E. Koshland, Jr., unpublished results). Because *che* and *fla* genes appear to be coordinately expressed, the absence of methylesterase activity in extracts from *fla*$^-$ strains supports the conclusion that the methylesterase is a *che* gene product.

In *E. coli* two genes, designated *tar* and *tsr*, code for 60,000-dalton methylated membrane proteins (9–11). When mutant strains defective in these genes were assayed for methylesterase activity, elevated levels were obtained (Table 2). In order to form a complex with its substrates, the methylesterase must interact with membranes. It is therefore possible that the activity detected in wild-type cell extracts represents only a fraction of the total methylesterase level within the cell, the remainder being bound to 60,000-dalton proteins within the membrane. This would explain the relatively high levels of methylesterase activity found in cells lacking a full complement of these membrane proteins. In this context, it should be noted that the *cheB* gene product in *E. coli* has been shown to be located in both membrane and soluble cell fractions (28).

Characterization of Methylesterase. The soluble fraction of wild type *S. typhimurium* was fractionated by Sephadex G-100 column chromatography to isolate the methylesterase. The activity was well separated from the bulk of the protein,

Table 3. Methylesterase activities in nonchemotactic (*che*$^-$) strains of *Escherichia coli*

Strain	Relevant genotype	Methylesterase activity,* %	Nongradient swimming behavior
RP437	Wild type	100	Random
RP4303	*cheA*$^-$	102	Smooth
RP4310	*cheB*$^-$	8	Tumbly
RP4792	*cheD*$^-$	64	Smooth
RP4305	*cheW*$^-$	64	Smooth
RP4306	*cheX*$^-$	55	Smooth
RP4315	*cheY*$^-$	59	Smooth
RP4318	*cheZ*$^-$	92	Tumbly

* The wild-type (RP437) extract catalyzed hydrolysis of 1.2% of the carboxyl methyl groups in methylated *S. typhimurium* ST1038 membranes per mg of extract protein per ml per hr.

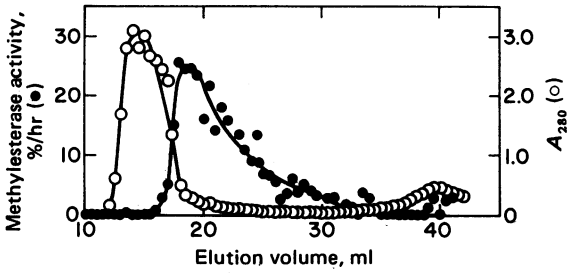

FIG. 3. Characterization of methylesterase by Sephadex G-100 column chromatography. An *S. typhimurium* ST1 soluble extract containing 25 mg of protein in 0.5 ml of 0.1 M sodium phosphate/1.0 mM EDTA buffer, pH 7.1, was chromatographed on a 1.2 × 47 cm Sephadex G-100 column equilibrated with the same buffer. Fractions (0.5 ml) were collected and assayed for methylesterase activity (release of methanol from membranes) as described in *Materials and Methods* (●); the absorbance at 280 nm of a 1:10 diluted aliquot of each fraction was also measured (○).

most of which eluted in the void volume (Fig. 3). The methylesterase elution profile was compared to that of proteins of known molecular weight to obtain an estimate of the molecular weight of the methylesterase; the result was a value of approximately 100,000. Because Silverman and Simon (26) have estimated that the *cheB* gene product has a polypeptide molecular weight of approximately 38,000, it seems likely that the methylesterase is an oligomer.

DISCUSSION

The results described above establish that one of the proteins of the chemotactic system in *S. typhimurium* and *E. coli* catalyzes the demethylation of the methylated 60,000-dalton membrane proteins involved in the chemotactic response. Of the nine chemotaxis genes in *Salmonella*, two have now been associated with enzymatic activities. Evidence has previously been presented which indicates that the *cheR* gene product functions as a methyltransferase (12), and, from the work described here, it seems likely that the *cheX* gene product acts as a protein methylesterase. In *E. coli* the situation appears to be completely analogous: the *E. coli cheX* gene is responsible for the expression of methyltransferase,* and the *E. coli cheB* gene functions in expression of methylesterase activity.

The *cheT* gene product in *S. typhimurium* and the corresponding *cheZ* gene product in *E. coli* seem in some way involved in methylesterase function. This is suggested by the finding that at least one *cheT*$^-$ strain, ST171, is deficient in methylesterase activity. Furthermore, genetic studies in *E. coli* indicate that the *cheB* and *cheZ* gene products interact *in vivo* (11). Similar observations have been made with the corresponding genes, *cheX* and *cheT*, in *Salmonella*.* From these results we tentatively conclude that the *cheX* gene product is a protein methylesterase, and that this enzyme, in turn, interacts in some way with the *cheT* gene product.

It is interesting to compare the properties of the two classes of *che* mutants that are now known to be involved in methylation. Methyltransferase-deficient mutants (*cheR*$^-$ in *Salmonella* and *cheX*$^-$ in *E. coli*) are smooth swimming in the absence of a gradient; conversely, the methylesterase-deficient mutants (*cheX*$^-$ in *Salmonella* and *cheB*$^-$ in *E. coli*) are constantly tumbly under these conditions (8, 11). Increases in attractant concentrations cause a transient smooth swimming response in methylesterase-deficient mutants (11, 29), and increases in repellent concentrations cause a tumbling response in methyltransferase-deficient mutants (12). These results support the possibility that methylation is important in control of the time constants of response and adaptation (12, 19).

Reversible covalent protein modification reactions play a central role in the regulation of a variety of cellular processes (30–32). Enzymes that catalyze the transfer of methyl groups from *S*-adenosylmethionine to carboxyl groups on proteins have been found in every organism and tissue that has been examined (33). However, no protein methylesterase activities have been reported in these systems. This is not surprising in view of the paucity of information concerning the nature of the *in vivo* protein substrates of eukaryotic methyltransferases. Moreover, protein carboxyl methyl groups are often labile, nonenzymatically, even at neutral pH (34, 35), and the resulting high nonspecific background of spontaneous methyl ester hydrolysis would make it difficult to detect specific protein methylesterase activities. Methyltransferases have now been shown by Axelrod and coworkers (21, 36) to be involved in neural cell function, and preliminary evidence indicates that proteins modified in this way function in leukocyte chemotaxis (22). It seems likely that methylation and demethylation of protein carboxyl groups plays a role in regulation analogous to that of phosphorylation and dephosphorylation. If so, the demonstration of a protein methylesterase in the bacterial system may be a forerunner for the finding of similar enzymes in eukaryotic species.

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